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PRINCIPAL INVESTIGATOR: Bernard Kwabi-Addo, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

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14. ABSTRACT

PURPOSE. The Sprouty gene family negatively regulates growth factor-induced receptor tyrosine kinase signaling with a potential tumor suppressor function in cancer. I have demonstrated that Sprouty1 is down-regulated in human prostate cancer (PCa). The purpose of the present study is to characterize the molecular mechanisms regulating Sprouty1 expression in the human PCa. Results. I have carried out deletion analysis coupled with reporter gene assays to characterize Sprouty1 promoter activity. Electrophoretic mobility shift assays, chromatin immunoprecipitation and TranSignal protein-DNA array were used to demonstrate binding interaction of Transcription factors (TFs) with Sprouty1 promoter. I have also carried out DNA methylation analysis on 20 matched normal prostate tissues and tumor prostate tissues (at least 70% of tissue is carcinoma) in the 5' untranslated region of Sprouty1. The results of deletion analysis demonstrated a strong promoter activity in the proximal 0.3-kb region of Sprouty1 promoter. Several potential binding sites for transcription factors (TFs) such as: AP-1/2, CREB, EGR1, GATA1, and SP1 were found within this region. In addition, TranSignal protein-DNA array analysis showed differential activation of a number of transcription factors (TFs) in the normal and prostate cancer cell lines with the consensus binding sites of Sprouty1 promoter. Gene knockdown of one such TF family: GATA (2 and 4) induced Sprouty1 expression demonstrating transcriptional repression by this TF. I did not observe any significant methylation of the Sprouty1 promoter region in the normal or the tumor samples analyzed. CONCLUSION. My studies suggest that Sprouty1 is not regulated in human prostate cancer by epigenetic mechanisms. Transcriptional repression may therefore represent a key mechanism for down-regulation of Sprouty1 expression in prostate cancer.

15. SUBJECT TERMS
Sprouty1; transcriptional regulation; prostate cancer; Cis-acting elements, Reporter gene assay; DNA-methylation analysis

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INTRODUCTION

Prostate cancer (PCa) is the second most common malignancy and the second leading cause of cancer deaths in men in the United States. There is abundant evidence indicating that inappropriate activation of fibroblast growth factor receptor (FGFR) signaling plays a critical role in the initiation and progression of prostate cancer (for review see [1]). Sprouty was originally identified in Drosophila as a negative regulator of fibroblast growth factor (FGF) signaling during tracheal development [2]. Subsequent studies have shown Sprouty to be a general inhibitor of growth factor-induced receptor tyrosine kinase (RTK) signaling pathways involved in Drosophila development and organogenesis [3-5]. While Drosophila has only one Sprouty gene, at least four Sprouty homologues (Sprouty1-4) have been found in humans and mice [6-8]. Mammalian Sprouty inhibit growth factor-induced cell responses, by inhibiting the RTK-dependent Ras/mitogen-activated protein (MAP) kinase signaling pathway [9-16]. Several mechanisms for Sprouty inhibition of the RTK/Ras/MAP kinase pathway have been proposed, including blocking the interaction of the Grb2/SOS complex with the docking protein, FRS2 [17,18] or the inhibition of Raf [19,20]. Another characteristic of the Sprouty inhibitors is their regulation by growth factors in a negative feedback loop. Specifically, growth factors regulate both the level of Sprouty transcript [21] and in some systems, the recruitment of Sprouty proteins to the plasma membrane [22]. Given that Sprouty proteins can inhibit FGF signaling, they can potentially decrease the biological activities of FGFs in prostate cancer cells and inhibit their ability to promote cancer progression.

We have previously shown by immunohistochemical and quantitative real-time PCR analysis that Sprouty1 and Sprouty4 are down-regulated in a subset of prostate cancers

tissues when compared with normal prostate tissues [23,24]. McKie et al., [25] have observed that Sprouty2 expression is reduced in clinical prostate cancer tissues when compared with benign prostatic hyperplasia (BPH). The decrease in Sprouty expression in the human prostate cancer, despite elevated levels of FGF ligands and FGF receptors, implies a loss of an important growth regulatory mechanism in prostate cancers that may potentiate the effects of increased FGF and FGFR expression in prostate cancer tissues and may represent a novel mechanism that facilitates aberrant RTK signaling in prostate carcinogenesis.

We and others have shown epigenetic inactivation to be a key mechanism for silencing Sprouty proteins in the prostate. For instance, we have observed promoter methylation at Sprouty4 CpG islands in prostate cancer. More than half of all prostate cancer tissue DNAs were methylated in this region and methylation significantly correlated with decreased Sprouty4 expression. Furthermore the treatment of prostate cancer cells with 5-aza-dC reactivated Sprouty4 expression demonstrating that aberrant methylation represents a key mechanism of Sprouty4 down-regulation [26]. Similarly, extensive methylation of Sprouty2 has been observed in high grade invasive prostate cancers while control BPH tissues were predominantly unmethylated [27]. The suppressed Sprouty2 expression correlated with methylation of the CpG region in clinical samples indicating that methylation of the Sprouty2 promoter was the likely cause of its transcriptional inactivation in the prostate. However, promoter methylation does not seem to explain Sprouty2 inactivation in breast cancer. Cultured breast cancer cell lines in the presence of 5’Aza-2-deoxycytidine (5-aza-dC) a demethylation agent, did not reactivate the expression of Sprouty2 and only minimal and patient specific methylation of the

Sprouty2 CpG islands was found [28] indicating cancer-specific mechanisms of Sprouty down-regulation[29]. Therefore a full understanding of the molecular mechanisms regulating Sprouty1 must include knowledge of Sprouty1 transcription regulation. Thus, in the present study, I sought to investigate the relative contribution of transcriptional mechanisms to *Sprouty*1 gene inactivation in prostate cancer.

BODY

As outlined in my Statement of work, I seek to accomplish 3 main tasks during my 3 years of funding. In the past 2 years, I have made substantial progress on two of these tasks and work is currently underway to complete the last task in this final year of funding. A manuscript describing Transcriptional Inactivation of Sprouty1, a Negative Regulator of Fibroblast Growth Factor Signaling in Prostate Cancer is being reviewed by the Prostate Journal. A copy of this manuscript is attached and will be referred to below.

Task3: Characterization of transcription factor(s) responsible for interaction with Sprouty1 promoter (Months 18 – 36).

Identification of Transcription factors binding to Sprouty1 promoter region using TranSignal Protein/DNA Arrays, Electrotrophoretic Mobility Shift Assays and Chromatin Immunoprecipitation.

In task 2, I used 5'RACE analysis to localize the position of the human Sprouty1 transcription start site. The human Sprouty1 gene consists of two splice variants, 1a [30] and 1b [31] that maps to human chromosome 4q27-28 and 4q25-28 respectively. Each splice variant has 2 exons and one intron. Exon 1 encodes the 5'-untranslated region of

the cDNA, whereas exon2 encodes the remainder of the 5'-untranslated region, the entire open-reading frame and the entire 3'-untranslated region. While the splice variants share the same second exon, they have different first exons, located very close to each other on the same chromosome. I also carried out a series of unidirectional deletion analysis coupled with luciferase reporter gene assay in transient transfection assays to demonstrate a strong promoter activity in the proximal 0.3-kb region of *Sprouty*1b promoter, hereafter referred to as Sprouty1 promoter.

In task 3, I sought to characterize the transcription factors binding to the Sprouty1 promoter. Using a computer-based analysis (MatInspector software from Genomatix; www.Genomatix.de) I have found potential binding sites for several TFs including GATA1 [32], EGR2 [33], ZBP [34], ETS [35], HIC [36] and FKHD [37] in the proximal promoter region. The human and murine [38] *Sprouty*1 5'-flanking region upstream of their transcription start sites were aligned for sequence comparison. Over the entire 5'-flanking region of the human *Sprouty*1 promoter, only a very short region in *Sprouty*1b promoter (between -112 and +1 relative to the transcription) showed approximately 94% degree of homology with the mouse *Sprouty*1 promoter. As illustrated in Fig 1, Wilm's tumor (WT1) transcription factor binding sites: EGR1 and 3 [39], and WTE [40] are conserved between the two species. Interestingly, the nucleotide sequences immediately upstream from the EGR motif diverge in these species. Furthermore, I did not see any sequence homology between the human *Sprouty*1 promoter region and that of the published *Sprouty*2 [41] or *Sprouty*4 [42] promoters. The high sequence homology in the *Sprouty*1 promoter of the mouse and human indicates an evolutionary conserved mechanism(s) involving WT1 and EGR transcription factors in *Sprouty*1 gene regulation.

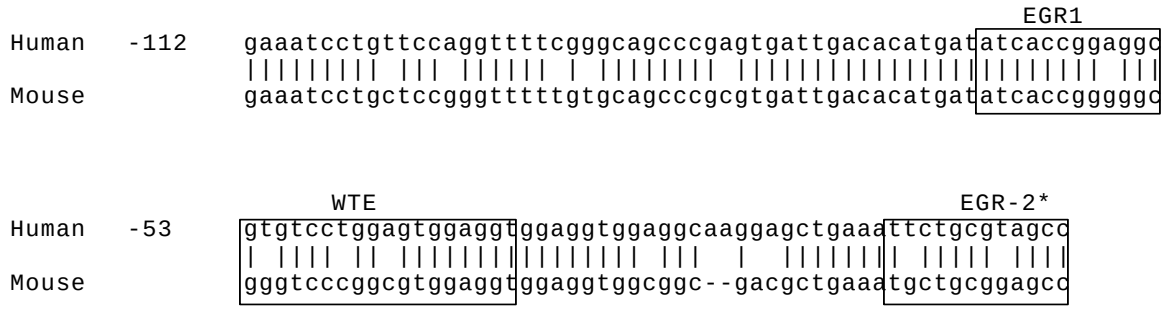


Fig 1. Alignment of sequence in the 5'-flanking region of human and murine Sprouty1 gene. The nucleotide sequences surrounding the transcription start site and the 5'-flanking region were compared. The putative binding sites for indicated transcription factors, which are conserved in both species, are boxed. An asterics (*) indicate core similarity of 1.000 with human sequence.

TranSignal Protein/DNA array analysis

In order to assess the activities of the transcription factors regulating Sprouty1 expression, I employed a protein/DNA array technology. This array is a high-throughput, DNA-based system that facilitates profiling the activities of multiple TFs in one assay (see www.panomics.com/pdf/PD_Array_1_with_ap.pdf) for a list of TFs binding sites on the array). To identify transcription factors whose activities might be altered in response to FGF2 stimulation, LNCaP cells were stimulated with or without recombinant FGF2 protein. As shown in Fig 2, the array analysis detected increased activities of several TFs in the LNCaP cells stimulated with FGF2 (Fig 2B) when compared with the unstimulated LNCaP cells (Fig 2A). In particular, the activity of EGR, ETS, GATA, HNF-4, PBX1 and SP1 which share consensus binding site on *Sprouty*1 proximal promoter were up-regulated in LNCaP cells stimulated with FGF2.

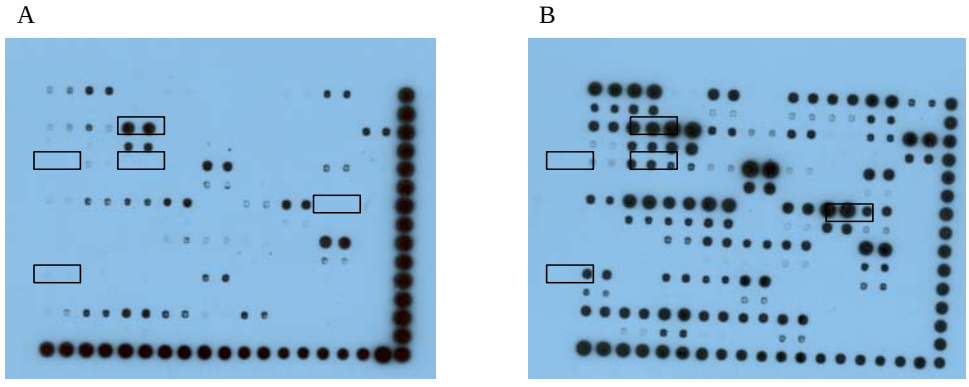
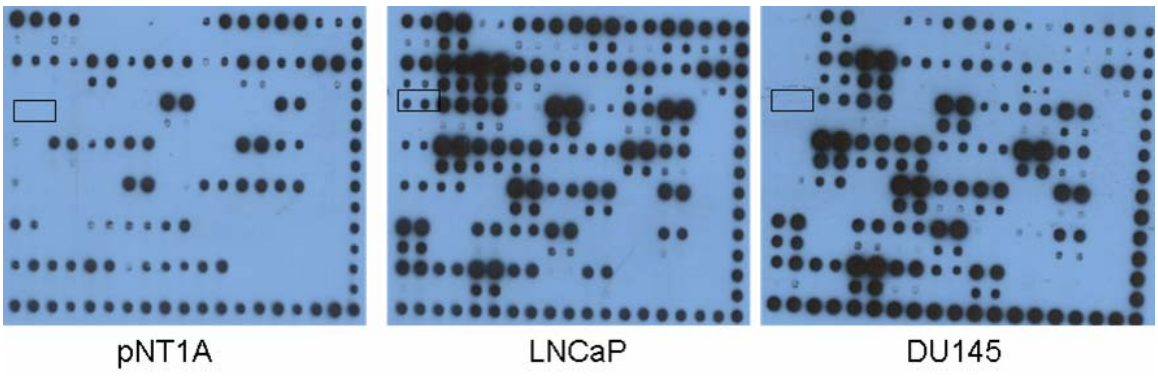


Fig 2. Comparison of FGF2 stimulated and unstimulated LNCaP cells with the protein/DNA array1. The array assay was performed using nuclear extracts from LNCaP cells grown in serum free medium (A) and LNCaP cells grown in serum free medium supplemented with FGF2 (20ng/ml). The boxed spots indicate differences in spots signal intensities in A and B. The dark spots along the right and bottom sides of the array indicate where biotinylated DNA has been spotted. These spots are intended for alignment.

Next, I compared the profile of the transcriptional activities of pNT1A, LNCaP and DU145 cells in response to FGF2 stimulation as shown in Fig 3A. My studies demonstrated differential activation of a number of transcription factors with consensus binding sites on *Sprouty*1 promoter in these cell lines. This includes AP-1/2, ARE, c-Myb, CREB, E2F1, EGR, ERE, GATA, Smad SBE, Stat 1-6, USF-1 and HSE following FGF2 treatment. The overall pattern of the response element occupancy indicates the activation of high number of transcription factors in the cancer cell lines (LNCaP and DU145) when compared to the normal pNT1A cell line. Of particular interest is the activation of transcriptional activator/repressor, GATA, specifically in the androgen dependent cell line LNCaP (indicated as boxed) which may be responsible for the low expression of Sprouty1 in LNCaP cells when compared to pNT1A and DU145 as determined by western blotting (Fig 3B).

A



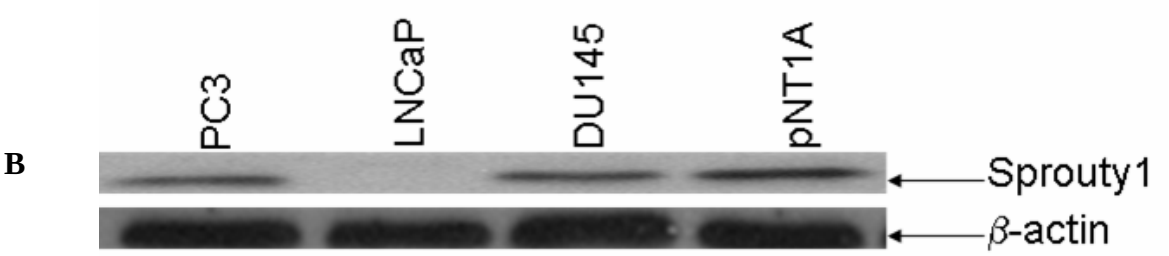


Fig 3. Comparison of FGF2 stimulated pNT1A, LNCaP and DU145 cells with the protein/DNA array1. A. The array assay was performed using nuclear extracts from pNT1A, LNCaP an DU145 cells grown in serum free medium and supplemented with FGF2 (20ng/ml). The boxed spots show different GATA signal intensities in the 3 cell lines. B. Protein extracts from the prostate cancer cell lines; PC3, LNCaP, DU145 and the immortalized normal prostate epithelial cell line pNT1A were analyzed by Western blotting with anti-Sprouty1 antibodies. In the LNCaP cells, the Sprouty1 protein expression is barely detectable. Loading control on the same filter with anti-β-actin antibody is shown in the lower panel.

Electrophoretic mobility shift assay

Because the protein/DNA array is a high-throughput method, the results require verification by a secondary assay. I therefore performed electrophoretic mobility shift assay (EMSA) using designed consensus radiolabelled oligonucleotide probes to recognize EGR1, PBX1, HNF-4 SP1 and nuclear extracts prepared from either LNCaP, PC-3 or pNT1A. Since all three cell lines demonstrated a similar band-shift pattern with each probe, only results using nuclear extracts from LNCaP cells were shown in Fig 4. Three protein-DNA complexes (C1, C2 and C3) were formed with each of the oligonucleotide probes. These complexes represented sequence-specific interactions of proteins with this region, since the addition of 100-fold molar excess of the corresponding unlabelled oligonucleotide probe was able to compete away these complexes. To characterize these complexes further, supershift EMSA was conducted using specific antibodies. The result showed that although a supershift band was not clearly identified, addition of anti-SP1, clearly abrogated the formation of C2, whereas supershift with anti-PBX1 and anti-HNF4 reduced the signal intensity of the respective C2 complex suggesting that the C2 complex is formed with SP1, PBX1 and HNF4 respectively. I did not see any significant effect of anti-EGR on the protein-DNA complexes. However, when the EGR1 consensus binding sequence was mutated (Mut

EGR1), we observed a new complex migrating very close with complex C2. Cold competition assay with wild-type EGR1 oligonucleotide competed out complex C2 totally but only partially competed the new complex. Furthermore, supershift assay successfully competed C2. This indicates that EGR1 protein preferentially recognize and interact with the wild-type EGR1 consensus binding sequence.

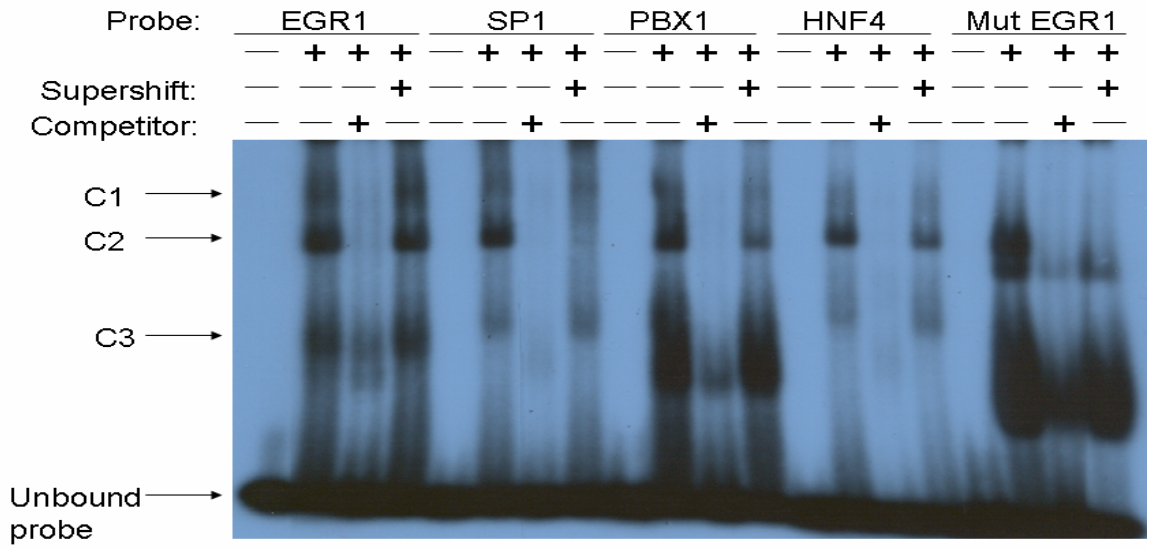


Fig 4. Analysis of TFs by electrophoetic Mobility Shift Assay. The analysis includes EGR1, SP1, PBX1 and HNF4. Radiolabelled double-stranded DNA oligonucleotides (probes) were incubated with or without nuclear extracts from LNCaP cells. Protein-DNA complex is indicated (C1, C2, C3), free or unbound probe is indicated at the bottom. Specificity of DNA-protein complex was investigated using 100 fold molar excess of corresponding unlabelled probe shown as competitor or the corresponding antibody shown as supershift.

Chromatin immunoprecipitation (ChIP)

I next studied whether these TFs bound to the *Sprouty*1 promoter *in vivo* using ChIP assay. Fig 5 showed that indeed these TFs bound to *Sprouty*1 promoter *in vivo* as demonstrated by the same PCR product in the assay precipitation with different antibodies compared to the Anti-acetyl-Histone H4 antibody control (positive control). Conversely precipitation with normal goat IgG (negative control) did not show any

binding. These studies clearly demonstrate that *Sprouty*1 proximal promoter region contain several sequence motifs (i.e., EGR, GATA, SP1, PBX1 and HNF4) which are specifically recognized by known as well as uncharacterized transcription factors and are functionally important and likely to be responsible for driving the basal transcription of the *Sprouty*1 gene.

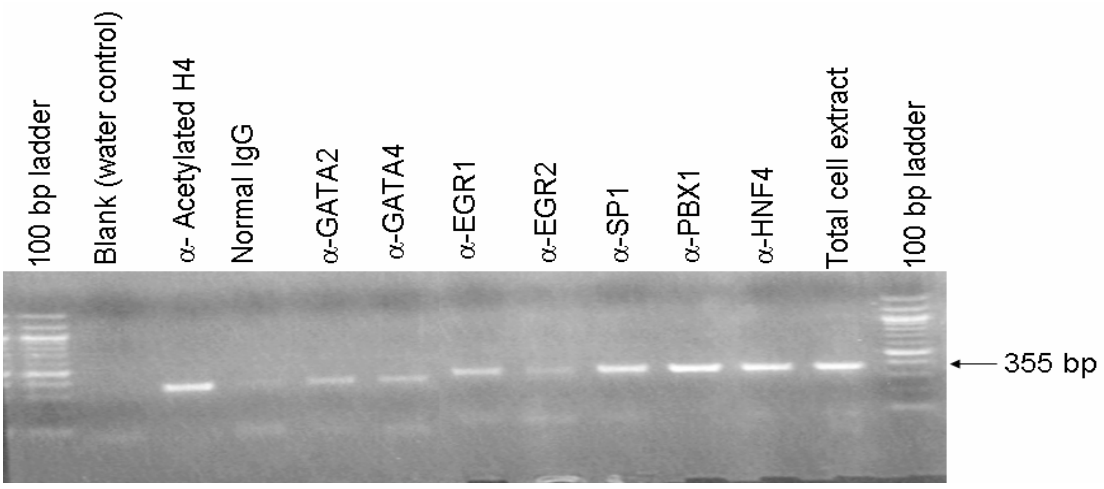


Fig. 5. Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays shows in vivo binding of different antibodies to the proximal *Sprouty*1b promoter. Anti-acetyl-Histone H4 antibody binding to DNA is used as a positive control (lane 3). Lanes 2 and 4 shows no amplification in the water and the normal IgG negative controls respectively.

Impact of EGR and GATA activity on Sprouty1 expression

To verify the involvement of EGR and GATA transcriptional activity in regulating Sprouty1 expression, I transiently transfected LNCaP cells with siRNA duplexes corresponding to EGR1, EGR2, GATA2 and GATA4. Western blot analysis were performed using Sprouty1 antibody and total cell lysates in order to examine the silencing effect of the EGR-1, EGR-2, GATA-2 and GATA-4 siRNA transfections on Sprouty1 protein expression. The Western blot signals were quantified and expressed relative to LNCaP cells transfected with scrambled siRNA oligos (negative control; data

not shown). Figure 6 shows that when compared with the LNCaP cells transfected with the scrambled siRNA oligos, LNCaP cells transfected with EGR1 siRNA (100 mM) showed a slight increase in Sprouty1 protein expression level. Transfection of EGR2 siRNA did not show a significant effect on Sprouty1 expression. On the order hand, when LNCaP cells were transfected with GATA2 siRNA (100 mM) and GATA4 siRNA (100 mM), there were approximately 4 and 3 fold increases in Sprouty1 protein expression respectively. The observed Sprouty1 expression levels were in response to 39%, 41%, 52% and 58% reduction of EGR1, EGR2, GATA2 and GATA4 mRNA expression respectively as determined by quantitative RT-PCR (data not shown). My data indicates that the blockade of EGR1 and EGR2 by small inhibitory RNA does not significantly affect Sprouty1 protein expression whereas the blockade of GATA2 and 4 can dramatically increase Sprouty1 protein expression.

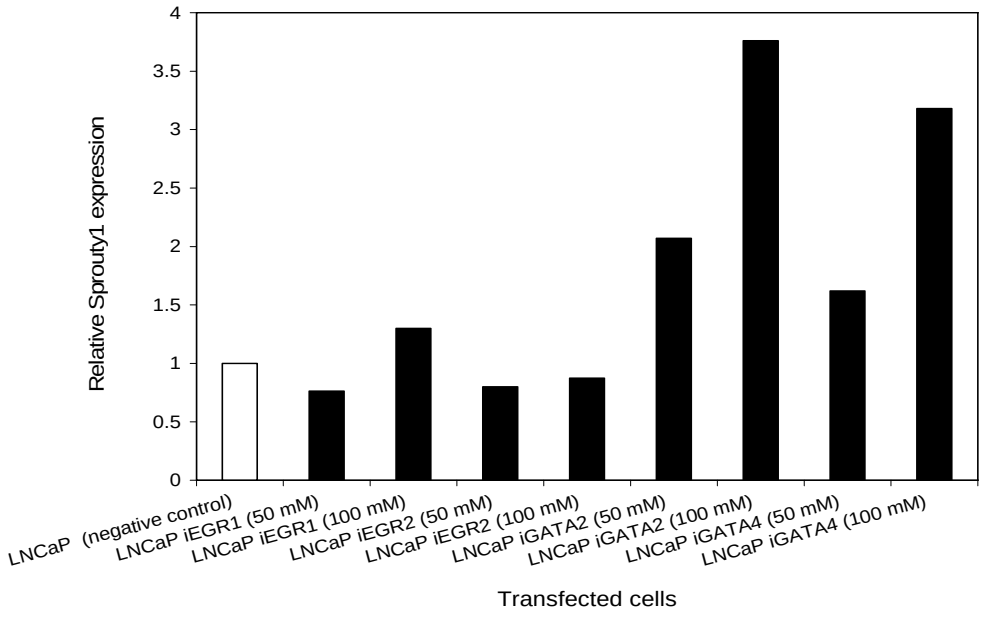


Fig 8. siRNA knock-down of EGR and GATA and Sprouty1 expression in LNCaP cells. The LNCaP cells were transiently transfected with either EGR1, EGR2, GATA2, or GATA4 siRNA duplexes for 72 hours. Total protein extracts from the transfected cells were used in western blotting with anti-Sprouty1

antibody. The Western blot signals were quantified using NucleoVision imaging workstation and calculated as the ratio of Sprouty1 protein to β-actin protein. For each transfection, the Sprouty1 expression level was expressed as a ratio relative to LNCaP cells transfected with scrambled siRNA oligos (negative control; where the Sprouty1 expression in the negative control was set at 1).

Methylation analysis of Sprouty1 promter by pyrosequencing.

To investigate DNA methylation of Sprouty1 promoter, we used pyrosequencing to quantitatively measure DNA methylation of bisulfite modified genomic DNA from 20 pairs of matched normal and tumor prostate tissue samples. Pyrosequencing was used to examine CpG islands of Sprouty1a and 1b promoters. A typical example of bisulfite methylation profiles presented as pyrogram is shown for Sprouty1a (Fig 7). The result does not demonstrate a significant level of methylation in the normal or tumor prostate tissues suggesting that the Sprouty1 promoter is not regulated by methylation in prostate cancer.

A. B.

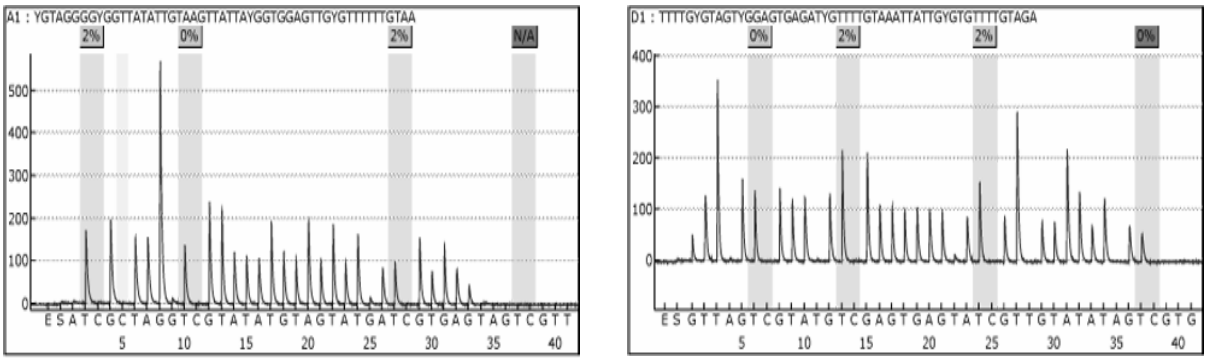


Fig. 7. Examples of CpG analysis Pyrogram traces. Normal prostate tissue (A) and tumor prostate tissue (B) were analysed by pyrosequencing. The gray shaded bars indicate region of C-to-T polymorphic sites. Analysis does not demonstrate a significant level of methylation at all 4 CpG sites.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of transcription factor binding interaction with Sprouty1 promoter in vitro

- Quantitative DNA methylation analysis by pyrosequencing suggests that DNA methylation does not play a role in Sprouty1 regulation in human prostate tissues.

- Demonstrate that knockdown of GATA (2 & 4) transcription factors by siRNA inhibition in prostate cancer cell line (LNCaP) restored Sprouty1 expression suggesting that transcriptional repression may be a key mechanism for Sprouty1 regulation in prostate cancer.

REPORTABLE OUTCOMES

- **Manuscript submitted for publication in the Prostate Journal**

- **Presentation-AACR Annual Meeting: Profiling the transcriptional regulation of Sprouty1, a negative regulator of growth factor signaling in androgen dependent and independent human prostate cancer cells. Kwabi-Addo et al., (2006). Washington D.C, (Abstract).**

CONCLUSION

The Sprouty gene family functions as negative regulators of receptor tyrosine kinase signaling. I have identified functional regions of the human *Sprouty*1 gene promoter, which are responsible for constitutive gene expression. DNA methylation analysis of the Sprouty1 promoter region did not show any significant methylation in matched normal and tumor prostate tissue samples suggesting that DNA methylation is not responsible for Sprouty1 downregulation. Thus, my data from the transcriptional regulation of *Sprouty*1 in prostate cancer cell lines implies that transcriptional repression may represent a key mechanism for the down-regulation of Sprouty1 expression in human prostate cancer.

Complete understanding of the molecular mechanisms controlling Sprouty1 expression may prove useful in elucidating the regulation of growth factor signals in prostate cancer which may in turn provide an attractive new target approach for therapeutic intervention

that may modulate a large number of potential growth promoting stimuli, including multiple growth factors and their receptors.

FUTURE WORK WILL FOCUS ON:

1) Complete the evaluation of GATA and EGR transactivation of the Sprouty1 promoter in human prostate cancer cells by mutating the EGR and GATA binding sites within the Sprouty1-luciferase reporter gene using site-directed mutagenesis in order to ascertain whether such mutations would alleviate GATA or EGR mediated transaction of the Sprouty1 activity in vitro.
2) Complete characterization of the highly conserved EGR binding site of human Sprouty1 promoter region. I did not observed a significant effect of EGR1 and EGR2 knockdown on Sprouty1 expression. Comparison of the amino acid sequence of WT1 and EGR1 revealed a high degree of similarity [43] and the results of a number of transient transfection studies have demonstrated WT1 repression of promoters responsive to EGR1 [44,45] suggesting that there may be a reciprocal expression between these two protein. I will investigate siRNA mediated gene knockout of WT1 and Sprouty1 expression in prostate cancer cells and also the effect of WT1 over-expression and Sprouty1 expresion to ascertain whether WT1 may be more important than EGR in human *Sprouty*1 regulation.
3) Investigate in vitro molecular interaction of TFs and Sprouty1 promoter. DNA methylation analysis does not appear to play a role in the down-regulation of Sprouty1 in prostate cancer. However, histone deactylases (HDACs) are known to act as repressors in the regulation of expression of many genes. To determine

whether histone deacetylases might be involved in Sprouty1 repression in prostate cancer, I will carry out transient transfection assays using plamid vectors encoding for transcription factors such as SPI that are able to bind GC-rich cis-elements and examine them in the presence and in the absence of trichostatin A (TSA), a specific inhibitor of histone deacetylases.

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134 Profiling the transcriptional regulation of Sprouty1, a negative regulator of growth factor signaling in androgen dependent and independent human prostate cancer cells

Bernard Kwabi-Addo, *Chengxi Ren*, *Michael Ittmann*. Baylor College of Medicine, Houston, TX.

Sprouty1 is a negative regulator of growth factor signaling with a potential tumor suppressor function in prostate cancer. In the majority of cancer samples examined, a significant loss of expression of Sprouty1 has been detected. Global demethylation appears to induce the expression of Sprouty1 whereas gene promoter methylation appears to be responsible for Sprouty1 reduced expression in same cancer samples. However, the mechanism for reduction is unknown in other samples. Mutation in the Sprouty1 coding sequence does not appear to be involved. In this report, we studied the regulation of Sprouty1 and cloned and functionally characterized the Sprouty1 promoter region. The Sprouty1 promoter lacks a TATA box and has a GC-rich region. Deletion mapping in combination with promoter activity assay showed that multiple cis-elements are involved in the transcriptional regulation of Sprouty1. To identify the transcription factors that are activated or repressed in response to fibroblast growth factor (FGF2) stimulation of Sprouty1 expression, we used a novel protein-DNA interaction based method, TranSignal protein-DNA array, to profile binding interaction of transcription factors from a pool of 54 unique transcription factor binding sequences using crude nuclear extract from the androgen dependent LNCaP cells, the androgen independent DU145 cells and the normal, pNT1a cells. Our studies showed differential activation of a number of transcription factors with consensus binding sites on Sprouty1 promoter in these cell lines. This includes AP-1/2, ARE, c-Myb, CREB, E2F1, EGR, ERE, GATA, Smad SBE, Stat 1-6, USF-1 and HSE following FGF2 treatment. The overall pattern of the response element occupancy indicates the activation of high number of transcription factors in the cancer cell lines compared to the normal cell line suggesting possible induction of transcription factors to regulate Sprouty1 expression. Of particular interest is the activation of transcriptional activator/repressor, GATA, specifically in the androgen dependent cell line LNCaP which may be responsible for the low expression of Sprouty1 in LNCaP cells when compared to pNT1A and DU145. Our approach to defining the DNA and protein components that dictate the expression of Sprouty1 may provide a strong basis for the design and development of therapeutic targets to up-regulate Sprouty1 expression as a means of controlling abnormal prostate growth.

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Transcriptional Inactivation of Sprouty1, a Negative Regulator of Fibroblast Growth Factor Signaling in Prostate Cancer

Bernard Kwabi-Addo*, Chengxi Ren and Michael Ittmann

Department of Pathology, Baylor College of Medicine, Houston, Texas.

PURPOSE. Sprouty1 is a negative regulator of fibroblast growth factor signaling with a potential tumor suppressor function in prostate cancer (PCa). Sprouty1 is downregulated in human PCa and Sprouty1 expression can markedly inhibit PCa proliferation in vitro. In this report, we investigated the transcriptional regulation of *Sprouty*1 in human PCa cells.

EXPERIMENTAL DESIGN. Deletion analysis coupled with reporter gene assays were used to characterize *Sprouty*1 promoter activity. Electrophoretic mobility shift assays, chromatin immunoprecipitation and TranSignal protein-DNA array were used to demonstrate binding interaction of Transcription factors (TFs) with *Sprouty*1 promoter.

RESULTS. Deletion analysis showed a strong promoter activity in the proximal 0.3-kb region of *Sprouty*1 promoter. Several potential binding sites for transcription factors (TFs) such as: AP-1/2, CREB, EGR1, GATA1, and SP1 were found within this region. In addition, TranSignal protein-DNA array analysis showed differential activation of a number of transcription factors (TFs) in normal and prostate cancer cell lines with the consensus binding sites on *Sprouty*1 promoter. Gene knockdown of one such TF family: GATA (2 and 4) induced Sprouty1 expression demonstrating transcriptional repression by this TF.

CONCLUSION. Our data suggests that transcriptional repression may represent a key mechanism for down-regulation of Sprouty1 expression in prostate cancer cell lines.

KEY WORDS: Sprouty1; transcriptional regulation; prostate cancer; Cis-acting elements, Reporter gene assay

*Correspondence to:

Bernard Kwabi-Addo, PhD,

Department of Pathology,

Baylor College of Medicine

1 Baylor Plaza Houston TX 77030

Tel: 713 798 3162

Fax: 713 798 5838

E-mail: bernardk@bcm.edu

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INTRODUCTION

Prostate cancer (PCa) is the second most common malignancy and the second leading cause of cancer deaths in men in the United States. There is abundant evidence indicating that inappropriate activation of fibroblast growth factor receptor (FGFR) signaling plays a critical role in the initiation and progression of prostate cancer (for review see [1]). Sprouty was originally identified in Drosophila as a negative regulator of fibroblast growth factor (FGF) signaling during tracheal development [2]. Subsequent studies have shown Sprouty to be a general inhibitor of growth factor-induced receptor tyrosine kinase (RTK) signaling pathways involved in Drosophila development and organogenesis [3-5]. While Drosophila has only one Sprouty gene, at least four Sprouty homologues (Sprouty1-4) have been found in humans and mice [6-8]. Mammalian Sprouty inhibit growth factor-induced cell responses, by inhibiting the RTK-dependent Ras/mitogen-activated protein (MAP) kinase signaling pathway [9-16]. Several mechanisms for Sprouty inhibition of the RTK/Ras/MAP kinase pathway have been proposed, including blocking the interaction of the Grb2/SOS complex with the docking protein, FRS2 [17,18] or the inhibition of Raf [19,20]. Another characteristic of the Sprouty inhibitors is their regulation by growth factors in a negative feedback loop. Specifically, growth factors regulate both the level of Sprouty transcript [21] and in some systems, the recruitment of Sprouty proteins to the plasma membrane [22]. Given that Sprouty proteins can inhibit FGF signaling, they can potentially decrease the biological activities of FGFs in prostate cancer cells and inhibit their ability to promote cancer progression.

We have previously shown by immunohistochemical and quantitative real-time PCR analysis that Sprouty1 and Sprouty4 are down-regulated in a subset of prostate cancers tissues when compared with normal prostate tissues [23,24]. McKie et al., [25] have observed that Sprouty2 expression is reduced in clinical prostate cancer tissues when compared with benign prostatic hyperplasia (BPH). The decrease in Sprouty expression in the human prostate cancer, despite elevated levels of FGF ligands and FGF receptors, implies a loss of an important growth regulatory mechanism in prostate cancers that may potentiate the effects of increased FGF and FGFR expression in prostate cancer tissues and may represent a novel mechanism that facilitates aberrant RTK signaling in prostate carcinogenesis.

We and others have shown epigenetic inactivation to be a key mechanism for silencing Sprouty proteins in the prostate. For instance, we have observed promoter methylation at Sprouty4 CpG islands in prostate cancer. More than half of all prostate cancer tissue DNAs were methylated in this region and methylation significantly correlated with decreased Sprouty4 expression. Furthermore the treatment of prostate cancer cells with 5-aza-dC reactivated Sprouty4 expression demonstrating that aberrant methylation represents a key mechanism of Sprouty4 down-regulation [26]. Similarly, extensive methylation of Sprouty2 has been observed in high grade invasive prostate cancers while control BPH tissues were predominantly unmethylated [27]. The suppressed Sprouty2 expression correlated with methylation of the CpG region in clinical samples indicating that methylation of the Sprouty2 promoter was the likely cause of its transcriptional inactivation in the prostate. However, promoter methylation does not seem to explain Sprouty2 inactivation in breast cancer. Cultured breast cancer cell lines in the

presence of 5'Aza-2-deoxycytidine (5-aza-dC) a demethylation agent, did not reactivate the expression of Sprouty2 and only minimal and patient specific methylation of the Sprouty2 CpG islands was found [28] indicating cancer-specific mechanisms of Sprouty down-regulation[29]. Therefore a full understanding of the molecular mechanisms regulating Sprouty1 must include knowledge of Sprouty1 transcription regulation. Thus, in the present study, we sought to investigate the relative contribution of transcriptional mechanisms to *Sprouty*1 gene inactivation in prostate cancer.

MATERIALS AND METHODS

Cell Culture and antibodies

The human prostate cancer cell lines, PC3, DU145 and LNCaP, and the immortalized normal prostate epithelial cell line pNT1A were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) unless otherwise stated. Antibodies used for western blotting, electrophoretic mobility shift assays and chromatin immunoprecipitation were the following: anti-Sprouty1 (C-12) goat polyclonal IgG; anti-PBX1 (P-20) rabbit polyclonal IgG; anti-GATA1 (C-20) goat polyclonal IgG; anti-GATA2 (C-20) goat polyclonal IgG; anti-GATA4 (C-20) goat polyclonal IgG; anti-EGR1 (588) rabbit polyclonal IgG; anti-EGR2 (N-20) goat polyclonal IgG; anti-SP1 mouse monoclonal IgG; anti-HNF4a (C-19) goat polyclonal IgG were all purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-acetyl-Histone H4 rabbit antiserum was purchased from Upstate Biotech (Lake Placid, NY).

Rapid amplification of cDNA ends

Transcription start sites were identified by 5'-RACE following the protocol from the GeneRacer Kit (Invitrogen) and using the human fetal lung Poly(A)$^+$ RNA (250 ng; Clontech, Palo Alto, CA) to create RACEready cDNA. A PCR reaction was carried out using RACEready cDNA as template and GeneRacer RNA oligo (5'-CGACTGGAGCACGAGGACACTGA-3') as the forward primer and either the Sprouty1-specific primer Spry1ARACE (5'-CTTGTCT-TGGTGCTGTCCGAGGAGC-

AGGT-3') or Spry1BRACE (5'-CTGCAAAGCACGCAGTGGTTTGCAGAGCGGA-3') as the reverse primer and Advantage-GC Genomic Polymerase Mix (Clontech).The PCR conditions consisted of one cycle of 3 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 68°C, and 1 min at 72°C. PCR products were cloned into the pCR-Blunt II-TOPO vector (Invitrogen) followed by sequencing.

Construction of plasmids for promoter analysis

Progressive deletion constructs of *Sprouty*1a and *Sprouty*1b 5'-flanking regions were accomplished by unidirectional cloning of PCR fragments from the *Sprouty*1 5'-flanking region into the *Kpn*1/*Nhe*1 site of the promoterless and enhancerless firefly luciferase reporter vector pGL3-Basic (Promega). For each region the PCR fragments were generated using a common reverse primer and different forward primers. The *Kpn1* and *NheI* sites (showed in lowercase) were engineered into the 5' ends of forward and reverse primers, respectively. The numbers indicated after the primer sequences correspond to the distance in nucleotides from the 5-end of the sequence in uppercase to the 5'-most transcription start site determined by 5'-RACE. For *Sprouty*1a 5'flanking region: Forward (Fwd) 13 (-93) 5'-ggtaccTCCTACCACAGAGAGAGGGAGAAA-3'; Fwd12 (-133) 5'-ggtaccCCCTCCTGAGCTCATGGTAACCT-3'; Fwd 6 (-509) 5'-ggtaccCTTCTGGTTTGGAGCACAGTGCAAAG-3'; Fwd 5 (-1318) 5'ggtaccAGAAG-ACCTCCCGAGGTGGATGTTA-3'; Fwd3 (-2025) 5'-ggtaccCTGTCAATCACCGGG-AGC-3'; Reverse (+8) 5'-gctagcAATCCGCACTGAATAAATAGTTGAC-3'. For *Sprouty*1b 5-flanking region: Fwd2 (-70) 5'-ggtaccCATGATATCACCGGAGGCGTGT-

CCTG-3'; Fwd3 (-175) 5'-ggtaccGAGTCTGTAGGGCAACATTTCCAAGTTGG-3'; Fwd 4 (-233) 5'-ggtaccCTGCATTTGCAGAATTTTTAGAGGCAC-3'; Fwd 5 (-305) 5'-ggtacc-CACCAATCCTTTTAATTGAGATCGAC-3'; Fwd6 (-530) 5'-ggtaccCATATG-CTTATATTACATTTGCAGTAAGG-3'; Fwd 7 (-960) 5'-ggtaccGTTTTGCCAGACT-TTAAGCTACTCC-3'; Reverse (+50) 5'-gctagcGGGAATGTGCTGATAATCACTCG-3'. We used the Advantage Genomic PCR kit (Clontech) to amplify these promoter fragments and according to the manufacturer's protocol. The human genomic DNA was used as template. The PCR products were first cloned into TOPO TA vector (Invitrogen) and then excised by *Kpn*1 and *Nhe*1 digestion and subcloned into the pGL3-Basic vector. Every construct was sequenced to ensure correct orientation and sequence integrity.

Transient transfections

The PC3, DU145, LNCaP and PNT1A cells were seeded on a six-well tissue plates in RPMI-1640 medium and supplemented with 10% FBS and grown for 16-24 hours to 80% confluence. Next cells were transiently transfected with the individual luciferase reporter plasmid by using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's procedure. The pSV-β-galactosidase control vector (Promega) was cotransfected with various luciferase reporter plasmids into cells to normalize the variations in transfection efficiency. To investigate FGF2 stimulatory effects cells were grown in serum free medium supplemented with 1% ITS for 24 hours. Cells were then transfected with the individual reporter plasmids as above. After 24 hours post transfection, cells were stimulated with or without FGF2 (20 ng/ml) for an additional 24 hours. Each transfection was done in triplicate.

Reporter gene luciferase assay

Cells were lysed 48 hours posttransfection by freeze thaw (3 cycles) in luciferase reporter lysis buffer (Promega). The lysates were centrifuged at 12,000 g for 2 min to remove cell debris. The supernatant was used for both luciferase and β-galactosidase activity assays. Luciferase activity was determined by using a luciferase assay kit (Promega) according to the manufacturer's protocol and measured in a luminometer. The β-galactosidase activity was assayed using the β-galactosidase enzyme assay kit (Roche) according to the manufacturer' protocol. Variation in transfection efficiency was normalized by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The promoterless pGL3-Basic vector was used as negative control, and the pGL3-CMV plasmid (which has CMV promoter and enhancer to drive the luciferase gene) was used as positive control for each transfection assay. Each reporter gene assay was done in triplicate.

siRNA cell transfection

The LNCaP (1 X 10^6) cells were each transfected with either 50 or 100 nM of siGENOME SMART pool GATA2 siRNA, GATA4 siRNA, EGR1 siRNA and EGR2 siRNA (Dharmacon Inc.) using 60 μl of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Seventy-two hours post-transfection cells were harvested and total protein extracted used in western blot analysis as previously described [30].

Preparation of nuclear extracts

Nuclear extracts were prepared from PC3, DU145, LNCaP and PNT1A essentially as previously described by [31].

FGF2 induction studies

The PNT1A, LNCaP and DU145 (1X 10^6) cells were each placed in serum free medium for 24 hours. Cells were refed with serum free medium with 1% ITS (Sigma) with or without 20ng/ml of recombinant FGF-2 (R&D Systems, Minneapolis, MN) and incubated at 37°C for additional 24 h. Cells were then harvested and crude nuclear extracts used in protein/DNA array as described below. Each induction study was done in triplicate.

TranSignal™ Protein/DNA Arrays

Protein array assays were performed following the procedure from the TranSignal Protein/DNA array kit user manual (Panomics). Briefly, 20 μg of nuclear extract (2-5 μg/ml) was mixed with probe mix and the mixture incubated at 15°C for 30 min. The mixture was then loaded onto a 2% agarose gel and electrophoresed at 120V in 0.5% TBE for 20 min. The gel area from above the blue dye to the loading well, which represents the migration distance of protein/DNA complexes, was excised from the agarose gel. The DNA probes were recovered from the protein/DNA complexes and denatured at 95°C for 3 min before being hybridized to the array membrane at 42°C overnight. The membrane was washed twice in 2 X SSC/0.5% SDS at 42°C for 20 min and then twice in 0.1 X SSC/0.5% SDS at 42°C for 20 min. The membrane was then blocked with 1X Blocking buffer at room temperature for 15 min. The biotin-labeled probe was then detected with Streptavidin-HRP diluted 1:1000. After washing three times and equilibrating in buffer, the membrane was overlaid with lumino/enhancer and substrate for 5 min. The image was acquired using Hyperfilm™ ECL (2-15 min). Each protein/DNA array assay was repeated at least once.

Oligonucleotide probe preparation and Electrophoretic mobility shift assays (EMSA)

Oligonucleotide sequences were synthesized by Sigma Genosys. Oligonucleotide sequences encompassing binding sites for wild-type or mutant (mut) transcription factors were synthesized as sense (F) or antisense (R) strands: EGRF 5'-GGATCCAGCGGGGGCGAGCGGGGGCCA-3'; EGRR 5'-TGGCCCCCGCTCGCC-CCCGCTGGATCC-3'; mut- EGRF 5'-GGATCCATTTTTTTCGATTTTTTTCCA-3'; mutEGRR 5'-TGGAAAAAAATCGAAAAAAATGGATCC-3'; PBX1F 5'-CGAATTG-ATTGATGCACTAATTGGAG-3'; PBX1R 5'-CTCCAATTAGTGCATCAATCAATT-CG-3'; HNF4F 5'-ACAGGGTCAAAGGTCACGA-3'; HNF4R 5'-TCGTGACCTTGA-CCCTGT-3'; SP1F 5'-ATTCGATCGATCGGGGCGGGGCGAG-3'; SP1R 5'-CTCG-CCCCGCCCCGATCGAAT-3'. Each oligonucleotide sense strand was end-labeled with [γ-^{32}P] dATP (Amersham Biochemicals; 3000 Ci/mmol) and 1X polynucleotide kinase reaction buffer (Invitrogen). The labeled oligonucleotide was annealed to 50 fold molar excess of the complementary anti-sense strand by heating at 85^{o}C for 5 min and slowly cooling to room temperature. Unincorporated [γ-^{32}P] dATP were removed by purifying the probes using a G-25 (Fine) Sephadex Quick spin columns (Roche). The EMSAs were carried out as described previously [32]. Briefly, the ^{32}P-labeled oligonucleotide probes were incubated with or without nuclear extract in a total reaction volume of 25 µl containing the binding assay buffer (50 mM Tris-HCl, pH 7.4; 50 mM NaCl; 1 mg/ml BSA; 5µg/ml poly dI-dC; 20% glycerol). The reactions were started by the addition of nuclear extract (5 µl per reaction) and incubated at room temperature for 30 minutes. Competition reactions were pre-incubated in the binding buffer for 30 min at room

temperature with 100 fold molar excess of the corresponding unlabelled oligonucleotides followed by a 30 min incubation at room temperature with the labeled oligonucleotide. For supershift analysis 1 µl of antibody was preincubated with the binding buffer for 45 minutes at room temperature prior to the addition of the ^{32}P-labeled probe. The bound and free DNA were resolved by electrophoresis through a 5% polyacrylamide gel at 175 V in 0.5 X TBE at room temperature for 2.5 h. Dried gels were exposed to Kodak Bio-Max film at -80^{o}C with intensifying screens.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) were performed as described previously [33]. For PCR reaction, DNA solution (50 ng) was used as a template with *Sprouty*1b Fwd 5 (forward) and Rev (reverse) primers as described above.

Western blot analysis- Western blotting for Sprouty1 protein and quantitative analysis of Western blot signals has been previously described [34].

RESULTS

Genomic organization of the Sprouty1 gene

The human *Sprouty*1 gene consists of two splice variants, 1a [35] and 1b [36] that maps to human chromosome 4q27-28 and 4q25-28 respectively. Each splice variant has 2 exons and one intron. Exon 1 encodes the 5'-untranslated region of the cDNA, whereas exon 2 encodes the remainder of the 5'-untranslated region, the entire open-reading frame and the entire 3'-untranslated region. While the splice variants share the same second exon, they have different first exons, located very close to each other on the same chromosome (Fig 1). The use of alternative promoters does not result in protein isoforms because the variant 5' initial exons are joined to a common second exon that contains the translation initiation site. In order to identify the transcription start sites of *Sprouty*1a and *Sprouty*1b splice variants, we performed 5'-RACE using poly $(A)^+$ RNA from fetal human lung and a *Sprouty*1 specific primer. We observed multiple bands after amplification, the largest of about 275 bp (data not shown). Sequence analysis identified multiple transcription initiation sites within the region -315 to -305 nucleotides from the first ATG codon in a Kozak consensus sequence. The 5'-most start site found is located at nucleotide position 160026 of the published sequence (GenBank accession no. AC026402). Because this region corresponds to the 5'-UTR of Splice variant 1b, this may represent the corresponding promoter region. Using similar approach we identified the transcription start site for Splice variant 1a to be at nucleotide position 162754 in the same published sequence (GenBank accession no. AC026402).

Functional characterization of Sprouty1 promoter region

To localize the DNA elements that are important for promoter activity, we carried out a series of unidirectional deletion analyses of up to 2 kb and approximately 1 kb of the 5'-flanking region of *Sprouty*1a of *Sprouty*1b splice gene variants, respectively. Deletion fragments were generated by PCR and cloned into the promoterless pGL3-Basic, a luciferase reporter vector. Each resulting recombinant construct was then transiently transfected along with the internal control pSV β-galactosidase plasmid into prostate cancer cell lines; LNCaP, PC3 and DU145 and the immortalized normal prostate cell line pNT1A. After 48 h, cell extracts were prepared and luciferase activity was measured and normalized to β-galactosidase activity. As shown in Fig 2, the promoter activities demonstrated significant difference between Splice 1a (Fig 2A) and 1b (Fig 2B) variants. *Sprouty*1a promoter strength was between 2 to 5 fold above the basal level. Whereas *Sprouty*1b promoter activity was between 40 and 900 fold above basal level depending on the cell line. Furthermore, the reporter gene expression levels showed significant differences among the different prostate cell lines suggesting that cell-specific element(s) may be present in these sequences. Interestingly, the androgen-dependent cell line, LNCaP which expressed the least Sprouty1 protein level as determined by western blot analysis [37] showed the strongest promoter activity; expressing over 7 fold higher promoter activity than any of the other cell lines. The maximum promoter activity varied for each cell line: In LNCaP cells the maximum promoter activity was observed from the *Sprouty*1b Fwd3 (-175 to +50) construct. In the androgen independent prostate cancer cell lines PC3 and DU145, maximum promoter activity was observed from the *Sprouty*1b Fwd4 (-233 to + 50) and *Sprouty*1b Fwd6 (-530 to + 50) constructs respectively. In the immortalized normal prostate cell line pNT1A, maximum promoter activity was seen

with the *Sprouty*1b Fwd5 (-305 to + 50) construct. Because strong promoter activity was observed at the *Sprouty*1b promoter region, we believe this region has the transcriptional elements and enhancer sequence(s) necessary for *Sprouty*1 gene regulation. Therefore all subsequent promoter analysis was done at the *Sprouty*1b locus and is hereafter referred to as *Sprouty*1 promoter.

Effect of FGF2 stimulation on Sprouty1 promoter activity

Because Sprouty1 is a down-stream target for FGF signaling [38], we investigated the effect of FGF2 stimulation on *Sprouty*1 promoter activity. We analyzed the proximal promoter region of Sprouty1b by transiently transfecting each of 2 constructs namely; *Sprouty*1b Fwd3 (-175 to +50) and *Sprouty*1b Fwd5 (-305 to +50) into the prostate cell lines; pNT1A, LNCaP and Du145 in serum free medium and stimulating the cells with or without FGF2 and then cell extracts used in luciferase reporter assays as described above. Figure 3A shows that upon FGF2 stimulation, Fwd3 construct was induced in DU145 cells and repressed in LNCaP cells. FGF2 stimulation did not show any significant effect on Fwd3 construct activity in PC3 and pNT1A cell lines. Similarly, FGF2 stimulation induced Fwd5 activity in Du145 and also in pNT1A cells but repressed Fwd5 activity in LNCaP cells (Fig 3B). The data indicates that FGF2 mediated stimulation of transcriptional element(s) can either induce or repress *Sprouty*1 promoter activity depending on the cellular context.

Comparative sequence analysis of the Sprouty1 promoter locus

To further characterize the *Sprouty*1 promoter region, we searched for transcription factor binding sites using the MatInspector program [39]. We analyzed 2 kb of the genomic AC026402 sequence upstream of the *Sprouty*1b transcription start sites,

using computer-based analysis (MatInspector software from Genomatix; www.Genomatix.de). We found potential binding sites for several TFs including GATA1 [40], EGR2 [41], ZBP [42], ETS [43], HIC [44] and FKHD [45] in the proximal promoter region. The human and murine [46] *Sprouty*1 5'-flanking region upstream of their transcription start sites were aligned for sequence comparison. Over the entire 5'-flanking region of the human *Sprouty*1 promoter, only a very short region in *Sprouty*1b promoter (between -112 and +1 relative to the transcription) showed approximately 94% degree of homology with the mouse *Sprouty*1 promoter. As illustrated in Fig 4, Wilm's tumor (WT1) transcription factor binding sites: EGR1 and 3 [47], and WTE [48] are conserved between the two species. Interestingly, the nucleotide sequences immediately upstream from the EGR motif diverge in these species. Furthermore, we did not see any sequence homology between the human *Sprouty*1 promoter region and that of the published *Sprouty*2 [49] or *Sprouty*4 [50] promoters. The high sequence homology in the *Sprouty*1 promoter of the mouse and human indicates an evolutionary conserved mechanism(s) involving WT1 and EGR transcription factors in *Sprouty*1 gene regulation.

Identification of transcription factors that regulates Sprouty1 expression

In order to assess the activities of the transcription factors regulating Sprouty1 expression, we employed a protein/DNA array technology. This array is a high-throughput, DNA-based system that facilitates profiling the activities of multiple TFs in one assay (see www.panomics.com/pdf/PD_Array_1_with_ap.pdf for a list of TFs binding sites on the array). To identify transcription factors whose activities might be altered in response to FGF2 stimulation, LNCaP cells were stimulated with or without recombinant FGF2 protein. As shown in Fig 5, the array analysis detected increased

activities of several TFs in the LNCaP cells stimulated with FGF2 (Fig 5B) when compared with the unstimulated LNCaP cells (Fig 5A). In particular, the activity of EGR, ETS, GATA, HNF-4, PBX1 and SP1 which share consensus binding site on *Sprouty*1b Fwd3 promoter were up-regulated in LNCaP cells stimulated with FGF2. Next, we compared the profile of the transcriptional activities of pNT1A, LNCaP and DU145 cells in response to FGF2 stimulation as shown in Fig 6A. Our studies demonstrated differential activation of a number of transcription factors with consensus binding sites on *Sprouty*1 promoter in these cell lines. This includes AP-1/2, ARE, c-Myb, CREB, E2F1, EGR, ERE, GATA, Smad SBE, Stat 1-6, USF-1 and HSE following FGF2 treatment. The overall pattern of the response element occupancy indicates the activation of high number of transcription factors in the cancer cell lines (LNCaP and DU145) when compared to the normal pNT1A cell line. Of particular interest is the activation of transcriptional activator/repressor, GATA, specifically in the androgen dependent cell line LNCaP (indicated as boxed) which may be responsible for the low expression of Sprouty1 in LNCaP cells when compared to pNT1A and DU145 as determined by western blotting (Fig 6B).

Electrophoretic mobility shift assay

Because the protein/DNA array is a high-throughput method, the results require verification by a secondary assay. We therefore performed electrophoretic mobility shift assay (EMSA) using designed consensus radiolabelled oligonucleotide probes to recognize EGR1, PBX1, HNF-4 SP1 and nuclear extracts prepared from either LNCaP, PC-3 or pNT1A. Since all three cell lines demonstrated a similar band-shift pattern with each probe, only results using nuclear extracts from LNCaP cells were shown in Fig 7A.

Three protein-DNA complexes (C1, C2 and C3) were formed with each of the oligonucleotide probes. These complexes represented sequence-specific interactions of proteins with this region, since the addition of 100-fold molar excess of the corresponding unlabelled oligonucleotide probe was able to compete away these complexes. To characterize these complexes further, supershift EMSA was conducted using specific antibodies. The result showed that although a supershift band was not clearly identified, addition of anti-SP1, clearly abrogated the formation of C2, whereas supershift with anti-PBX1 and anti-HNF4 reduced the signal intensity of the respective C2 complex suggesting that the C2 complex is formed with SP1, PBX1 and HNF4 respectively. We did not see any significant effect of anti-EGR on the protein-DNA complexes. However, when the EGR1 consensus binding sequence was mutated (Mut EGR1), we observed a new complex migrating very close with complex C2. Cold competition assay with wild-type EGR1 oligonucleotide competed out complex C2 totally but only partially competed the new complex. Furthermore, supershift assay successfully competed C2. This indicates that EGR1 protein preferentially recognize and interact with the wild-type EGR1 consensus binding sequence.

Chromatin immunoprecipitation (ChIP)

We next studied whether these TFs bound to the *Sprouty*1 promoter *in vivo* using ChIP assay. Fig 7B showed that indeed these TFs bound to *Sprouty*1 promoter *in vivo* as demonstrated by the same PCR product in the assay precipitation with different antibodies compared to the Anti-acetyl-Histone H4 antibody control (positive control). Conversely precipitation with normal goat IgG (negative control) did not show any binding. These studies clearly demonstrate that *Sprouty*1 proximal promoter region

contain several sequence motifs (i.e., EGR, GATA, SP1, PBX1 and HNF4) which are specifically recognized by known as well as uncharacterized transcription factors and are functionally important and likely to be responsible for driving the basal transcription of the *Sprouty*1 gene.

Impact of EGR and GATA activity on Sprouty1 expression

To verify the involvement of EGR and GATA transcriptional activity in regulating Sprouty1 expression, we transiently transfected LNCaP cells with siRNA duplexes corresponding to EGR1, EGR2, GATA2 and GATA4. Western blot analysis were performed using Sprouty1 antibody and total cell lysates in order to examine the silencing effect of the EGR-1, EGR-2, GATA-2 and GATA-4 siRNA transfections on Sprouty1 protein expression. The Western blot signals were quantified and expressed relative to LNCaP cells transfected with scrambled siRNA oligos (negative control; data not shown). Figure 8 shows that when compared with the LNCaP cells transfected with the scrambled siRNA oligos, LNCaP cells transfected with EGR1 siRNA (100 mM) showed a slight increase in Sprouty1 protein expression level. Transfection of EGR2 siRNA did not show a significant effect on Sprouty1 expression. On the order hand, when LNCaP cells were transfected with GATA2 siRNA (100 mM) and GATA4 siRNA (100 mM), there were approximately 4 and 3 fold increases in Sprouty1 protein expression respectively. The observed Sprouty1 expression levels were in response to 39%, 41%, 52% and 58% reduction of EGR1, EGR2, GATA2 and GATA4 mRNA expression respectively as determined by quantitative RT-PCR (data not shown). Our data indicates that the blockade of EGR1 and EGR2 by small inhibitory RNA does not significantly

affect Sprouty1 protein expression whereas the blockade of GATA2 and 4 can dramatically increase Sprouty1 protein expression.

DISCUSSION

In the present study, we have characterized the 5'-flanking region of the human *Sprouty*1 gene which is responsible for its transcriptional regulation in cell culture. We used a combination of luciferase reporter gene assays from transiently transfected cells, protein/DNA array and electrophoretic mobility shift assays to identify the cis-elements within the human *Sprouty*1 promoter region that confers responsiveness to FGF signaling. Our protein/DNA array analysis of the transcriptional regulation of human Sprouty1 in response to FGF signal transduction showed that FGF signaling cascades targeted common, ubiquitously expressed transcription factors such as, the ETS, AP-1 and ATF/CREB proteins that are also targeted by a number of other signaling molecules besides FGF. We have also shown that FGF2 stimulation can either induce or repress *Sprouty*1 promoter activity, depending on the cell type. So how does FGF signaling utilizes such general factors for the differential activation of *Sprouty*1 promoter and consequently differential expression of Sprouty1 protein in different prostate cell lines. As reviewed by Dailey et al [51] one fundamental aspect to understanding the specificity of the transcriptional response to FGF signaling lies in the nature of the elements controlling transcriptional activation. Transcriptional enhancers and promoters are composite elements containing specific spatial arrangements of binding sites for multiple transcription factors and it is only upon the binding of particular combinations of these factors that transcriptional activation will occur. In addition, the transcription factors may either be ubiquitously expressed, cell type-specific, or need to be activated by signal

transduction. Thus, enhancer activity depends on the presence and/or activation of several transcription factors in a given cellular and signaling context, and their assembly into multiprotein complexes in which they can combinatorially and synergistically activate or repress transcription [52].

We have identified a highly conserved nucleotide binding site for the early growth response (EGR1) in the human and mouse *Sprouty*1 promoter region which underscore the importance of this motif in the regulation of the *Sprouty*1 promoter expression. In the human prostate, there is strong evidence to suggest that EGR1 overexpression is involved in prostate cancer progression [53]. For example, EGR1 expression levels are elevated in human prostate carcinomas in proportion to grade and stage. Whereas antisense oligonucleotides that block EGR1 function revert transformation of prostate cancer cells *in vitro* and delay prostate cancer progression *in vivo* [54]. We did not observed a significant effect of EGR1 and EGR2 knockdown on Sprouty1 expression under our experimental conditions. However, EGR binding site is the same for the Wilms tumor (WT1) transcription factor. Gross et al., [55] have shown that the mouse *Sprouty*1 gene was upregulated by WT1 in embryonic kidney. Also comparison of the amino acid sequence of WT1 and EGR1 revealed a high degree of similarity [56] and the results of a number of transient transfection studies have demonstrated WT1 repression of promoters responsive to EGR1 [57,58] suggesting that there may be a reciprocal expression between these two protein: WT1 may act as an antagonist of EGR1 or may be a tissue specific factor as demonstrated for its transactivator effect of Sprouty1 expression specifically in the mouse embryonic kidney [59]. Furthermore, WT1 over-expression suppressed prostate tumor growth in vivo [60] clearly demonstrating the antagonistic response of

WT1 and EGR1 for the same binding site. It is possible that WT1 may play a more important role in human *Sprouty*1 regulation than EGR however, this remains to be established.

Results of protein/DNA array analysis demonstrated differences in the binding activity of transcription factors in the normal prostate and prostate cancer cell lines. In particular, our results showed a greater amount of GATA DNA binding activity specifically in the androgen responsive LNCaP cells. This observation is collaborated with previous observation by Perez-Stable *et al*., [61] who also observed a greater amount of GATA DNA binding activity in hormone-responsive prostate cell lines compared with androgen-independent prostate cancer cell lines. Our gene knockdown studies has established critical transcriptional repression role for GATA in Sprouty1 expression suggesting that one way that increased GATA DNA binding activity might contribute to prostate carcinogenesis might be to repress Sprouty1 expression.

In summary, we have identified functional regions of the human *Sprouty*1 gene promoter, which are responsible for constitutive gene expression. DNA methylation analysis of the Sprouty1 promoter region did not show any significant methylation in prostate cancer cell lines or matched normal and tumor prostate tissue samples (data not shown) suggesting that DNA methylation is not responsible for Sprouty1 downregulation. Thus, our data from the transcriptional regulation of *Sprouty*1 in prostate cancer cell lines implies that transcriptional repression may represent a key mechanism for the down-regulation of Sprouty1 expression in human prostate cancer.

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Figure legends

Fig 1. Schematic representation of the *Sprouty*1 gene. Exons are shown as open-boxes and translational start site, ATG is shown as a thick black bar. Promoter region is shown as blackened arrows. The use of alternative promoters does not result in protein isoforms because the variant 5' initial exons are joined to a common second exon that contains the translation initiation site.

Fig 2. Progressive deletion analysis of the 5'-flanking region of splice variant 1a and 1b of the human Sprouty1 gene. The schematic diagrams represent a series of *Sprouty*1a (A) and 1b (B) gene constructs with variable 5'-ends as indicated. The constructs were all cloned into a luciferase reporter vector (pGL3-Basic). 1.6 μg of each luciferase construct and 0.64 μg of the internal control pSV-β-galactosidase expression plasmid were transfected into LNCaP, DU145, PC-3 and pNT1A cells. Cells were lysed 48 hours post-transfection. The luciferase activity was measured and normalized for transfection efficiency by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The relative luciferase activities are represented as fold induction with respect to that obtained in cells transfected with the empty control vector (pGL3-Basic). Data represents the mean of triplicate experiments.

Fig 3. Effect of FGF2 stimulation on Sprouty1 promoter activity. The *Sprouty*1b Fwd3 (A) and Fwd 5 (B) were each (1.6 μg) co-transfected with 0.64 μg of pSV-β-galactosidase plasmid into prostate cell lines in serum free medium. 24 hour post-

transfection, transfected cells were grown in serum free medium supplemented with or without FGF2 (20 ng/ml) for additional 24 hr. Cells were lysed 48 hours post-transfection. The luciferase activity was measured and normalized for transfection efficiency by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The relative luciferase activities are represented as fold induction with respect that obtained in cells transfected with the empty control vector (pGL3-Basic). Data represents the mean of triplicate experiments.

Fig 4. Alignment of sequence in the 5'-flanking region of human and murine Sprouty1 gene. The nucleotide sequences surrounding the transcription start site and the 5'-flanking region were compared. The putative binding sites for indicated transcription factors, which are conserved in both species, are boxed. An asterics (*) indicate core similarity of 1.000 with human sequence.

Fig 5. Comparison of FGF2 stimulated and unstimulated LNCaP cells with the protein/DNA array1. The array assay was performed using nuclear extracts from LNCaP cells grown in serum free medium (A) and LNCaP cells grown in serum free medium supplemented with FGF2 (20ng/ml). The boxed spots indicate differences in spots signal intensities in A and B. The dark spots along the right and bottom sides of the array indicate where biotinylated DNA has been spotted. These spots are intended for alignment.

Fig 6. Comparison of FGF2 stimulated pNT1A, LNCaP and DU145 cells with the protein/DNA array1. A. The array assay was performed using nuclear extracts from pNT1A, LNCaP an DU145 cells grown in serum free medium and supplemented with FGF2 (20ng/ml). The boxed spots show different GATA signal intensities in the 3 cell lines. B. Protein extracts from the prostate cancer cell lines; PC3, LNCaP, DU145 and the immortalized normal prostate epithelial cell line pNT1A were analyzed by Western blotting with anti-Sprouty1 antibodies. In the LNCaP cells, the Sprouty1 protein expression is barely detectable. Loading control on the same filter with anti-β-actin antibody is shown in the lower panel.

Fig 7. Analysis of TFs activated by protein/DNA array1. A: The analysis includes EGR1, SP1, PBX1 and HNF4. Radiolabelled double-stranded DNA oligonucleotides (probes) were incubated with or without nuclear extracts from LNCaP cells. Protein-DNA complex is indicated (C1, C2, C3), free or unbound probe is indicated at the bottom. Specificity of DNA-protein complex was investigated using 100 fold molar excess of corresponding unlabelled probe shown as competitor or the corresponding antibody shown as supershift. B. Chromatin immunoprecipitation assays shows in vivo binding of different antibodies to the proximal *Sprouty*1b promoter. Anti-acetyl-Histone H4 antibody binding to DNA is used as a positive control (lane 3). Lanes 2 and 4 shows no amplification in the water and the normal IgG negative controls respectively.

Fig 8. siRNA knock-down of EGR and GATA and Sprouty1 expression in LNCaP cells. The LNCaP cells were transiently transfected with either EGR1, EGR2, GATA2,

or GATA4 siRNA duplexes for 72 hours. Total protein extracts from the transfected cells were used in western blotting with anti-Sprouty1 antibody. The Western blot signals were quantified using NucleoVision imaging workstation and calculated as the ratio of Sprouty1 protein to β-actin protein. For each transfection, the Sprouty1 expression level was expressed as a ratio relative to LNCaP cells transfected with scrambled siRNA oligos (negative control; where the Sprouty1 expression in the negative control was set at 1).

Fig. 1

Fig. 2

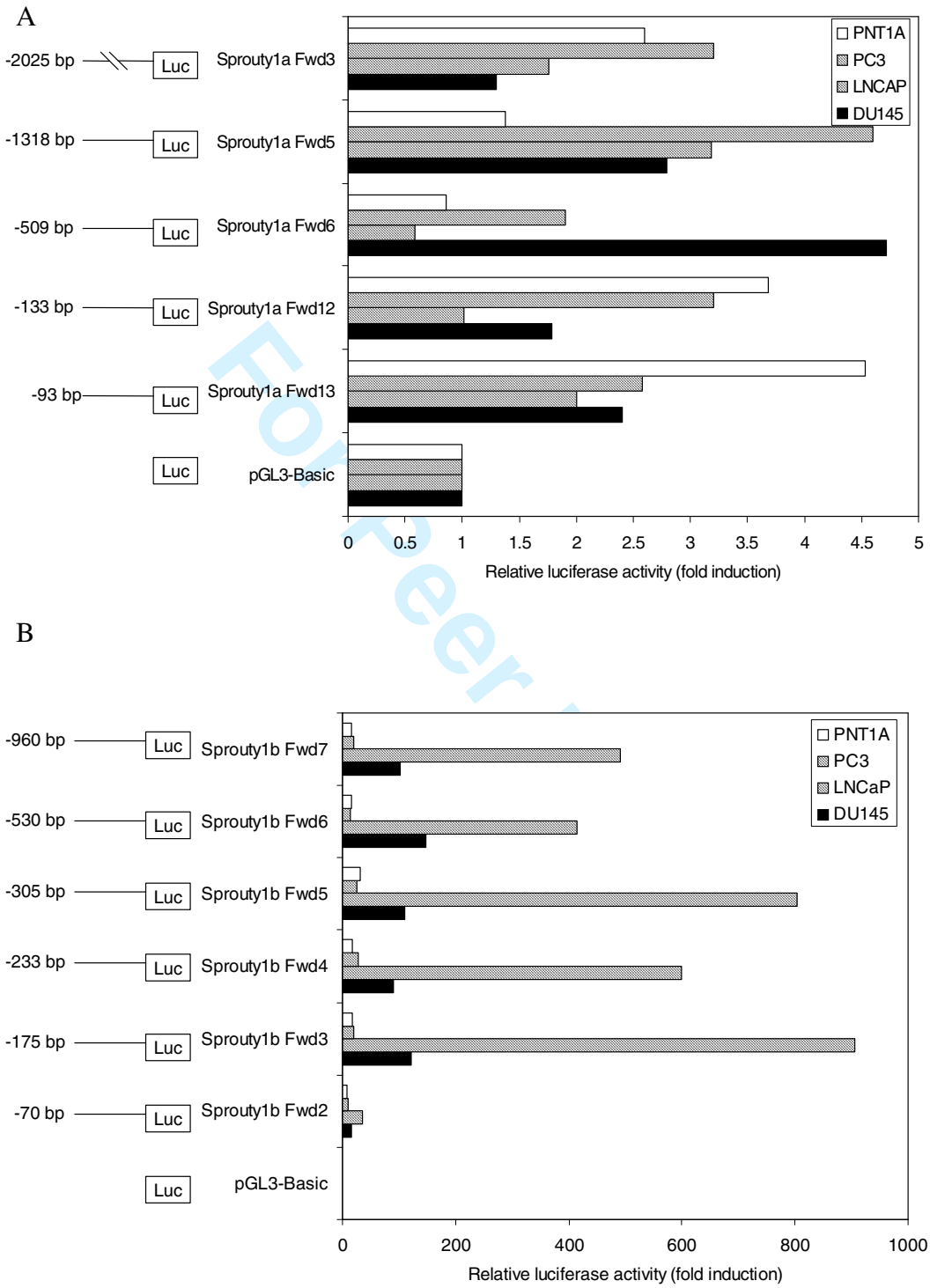


Fig. 3

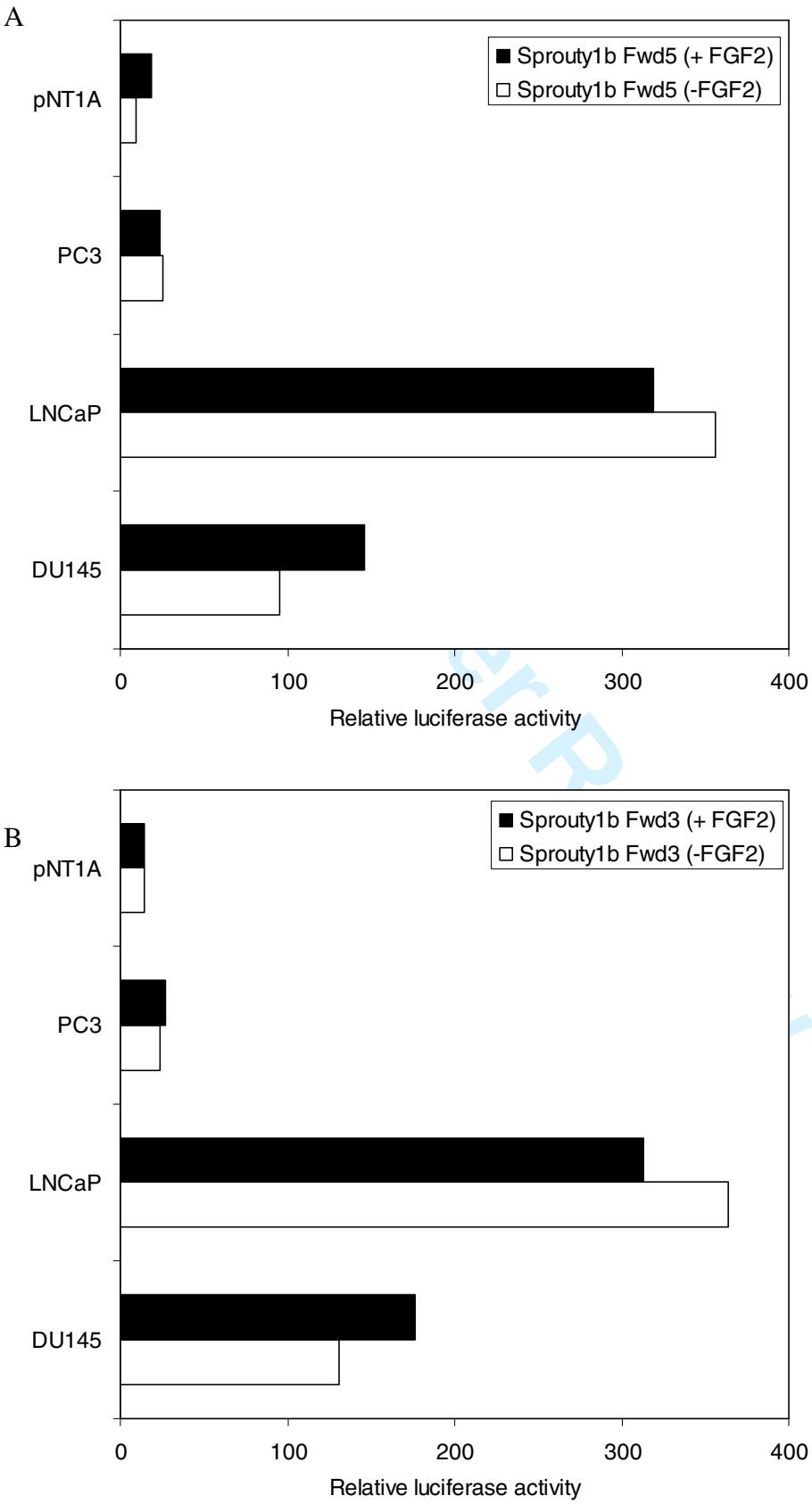

A
Sprouty1b Fwd5 (+ FGF2)
Sprouty1b Fwd5 (-FGF2)
pNT1A
PC3
LNCaP
DU145
0
100
200
300
400
Relative luciferase activity
B
Sprouty1b Fwd3 (+ FGF2)
Sprouty1b Fwd3 (-FGF2)
pNT1A
PC3
LNCaP
DU145
0
100
200
300
400
Relative luciferase activity

Fig. 4

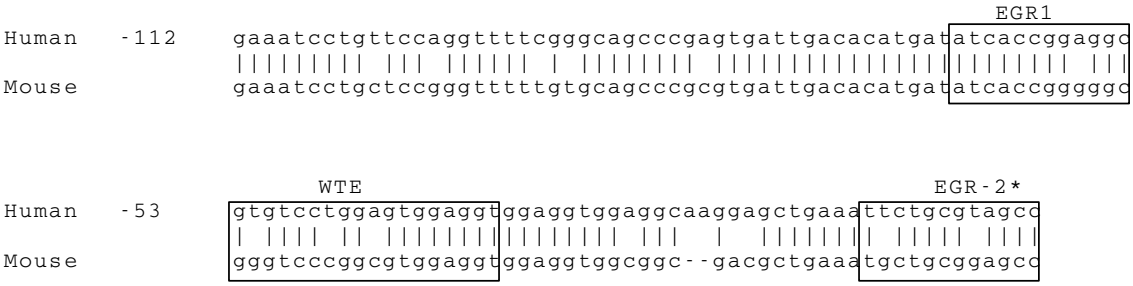
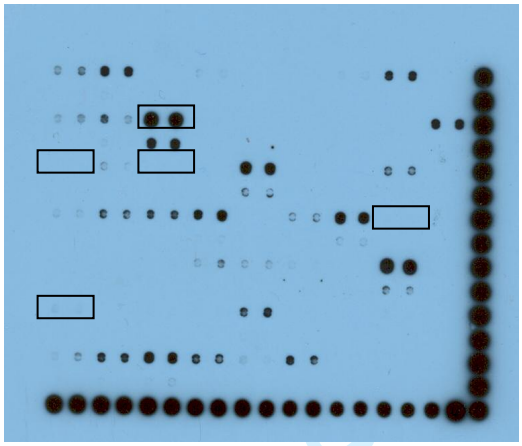


Fig. 5

A



B

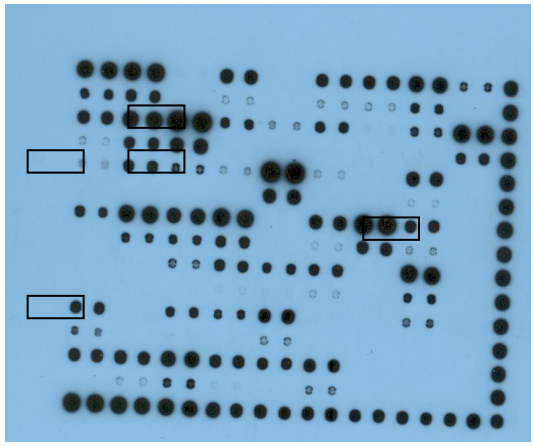
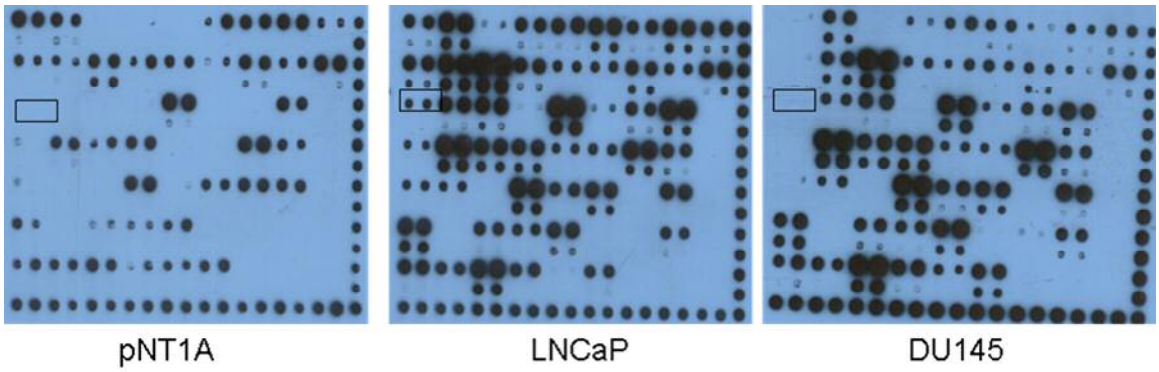


Fig. 6

A



B

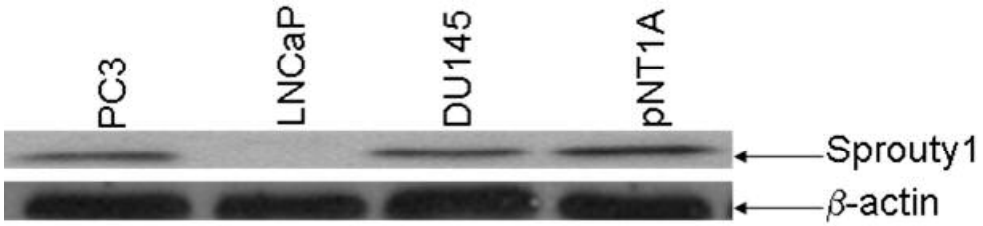
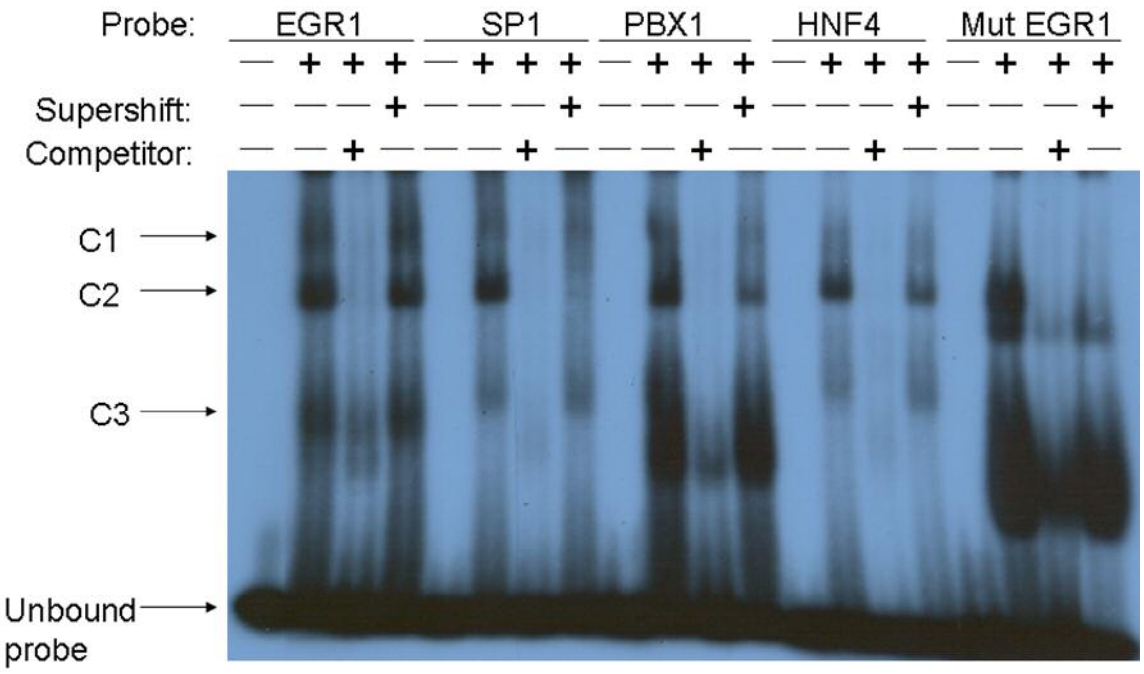


Fig. 7

A


Probe:
EGR1
SP1
PBX1
HNF4
Mut EGR1
Supershift:
Competitor:
C1
C2
C3
Unbound probe

B

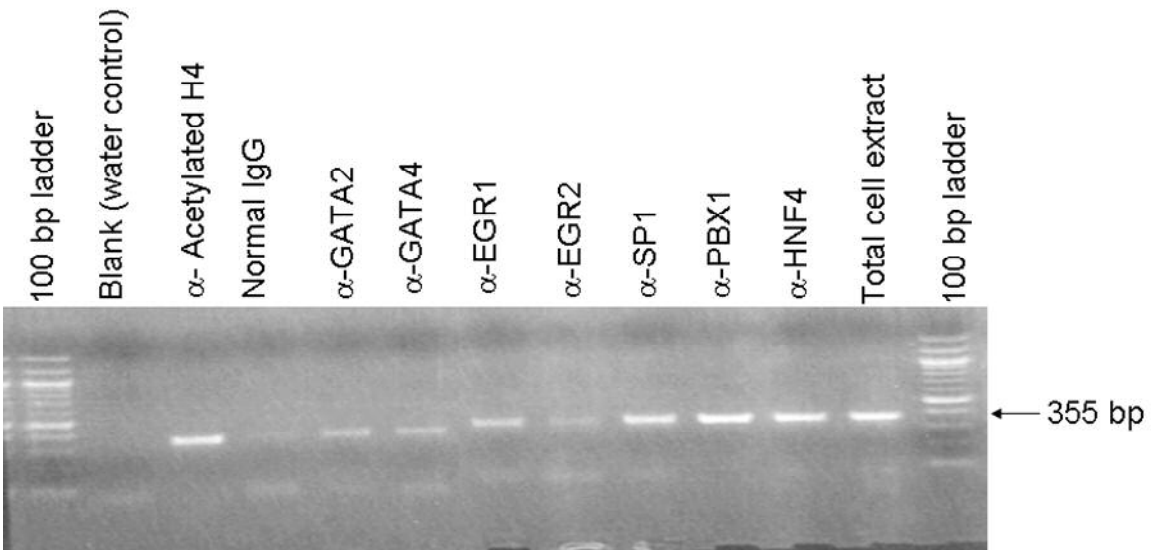

100 bp ladder
Blank (water control)
α- Acetylated H4
Normal IgG
α-GATA2
α-GATA4
α-EGR1
α-EGR2
α-SP1
α-PBX1
α-HNF4
Total cell extract
100 bp ladder
355 bp

Fig. 8